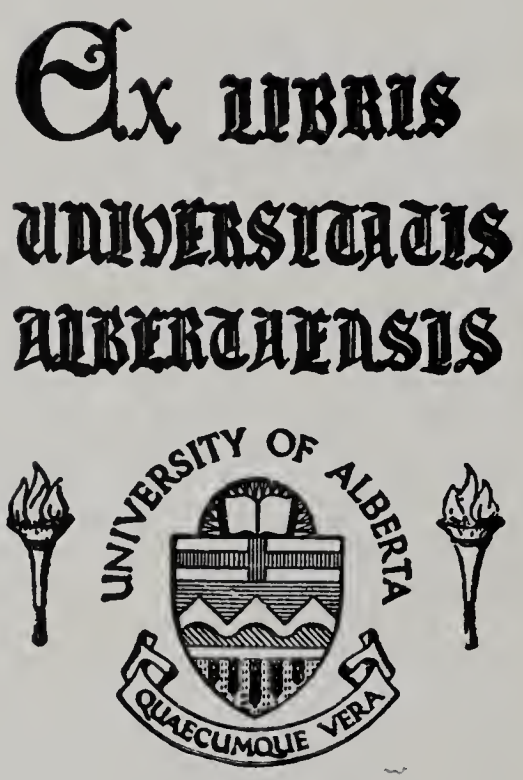

THE UNIVERSITY OF ALBERTA

STUDIES ON THE MICROBIAL MODIFICATION OF SOYBEANS FOR HUMAN CONSUMPTION

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF DAIRY AND FOOD SCIENCE

EDMONTON, ALBERTA

SEPTEMBER, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

STUDIES ON THE MICROBIAL MODIFICATION OF SOYBEANS FOR HUMAN CONSUMPTION

submitted by Yong Deng Hang in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A review has been made of the literature on the utilization of soybeans as human foods. Methods for the preparation of such important soybean food products as soybean milk, soybean curd or tofu, Chinese soybean cheese, soy sauce, miso and tempeh are described in detail.

A method has been investigated for the preparation of a soybean food product under controlled conditions using a lactic starter organism (Streptococcus thermophilus). Soybean milk was inoculated with starter bacteria and the curd cut, cooked, pressed, salted and ripened. Changes in pH, numbers of viable bacteria, moisture and nitrogen content were made during the manufacturing and ripening processes. The major role of lactic starter bacteria appeared to be acid production which is essential with this product. The low pH inhibited the growth of undesirable organisms which might have caused defects in the finished product.

Rennet extract added to the soybean milk did not reduce the time of coagulation, however, it improved the body and flavor of the soybean cheese. The addition of skimmilk resulted in a reduction of the time required from the addition of starter to the cutting of the curd. It would appear also that the flavor of the finished product was improved by the addition of skimmilk.

It is concluded that this new product could play a useful part in the nutrition of people in countries where soybean food products are readily acceptable.

ACKNOWLEDGEMENTS

This work was carried out during the tenure of a National Research Council Assistantship. I wish to express my sincere gratitute for this financial support.

I also wish to thank the academic and non-academic staff of the Department of Dairy and Food Science for their help throughout the course of this work, in particular Dr. Harold Jackson for his constant advice and encouragement.

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INTRODUCTION

Soybeans and products derived from them, according to Bailey et al. (1935), have for many centuries served as a primary source of protein in the diet of millions of Oriental people. During recent years, increased interest has developed in the nutritive value of soybeans and other sources of plant protein which may be used as human food partially to replace or extend animal proteins, such as milk, meat and eggs. Soybeans and their products continue to receive special emphasis in this field of application since they are easily adapted to a wide range of soil and climatic conditions and can be economically produced in many areas of the world. Since they also lend themselves well to shipping, they can be easily transported to areas where production is insufficient to meet local requirements.

Soybeans in their whole, unmodified form are considered to be relatively indigestable and have never been highly acceptable as a food. In the Oriental countries, they are either fractionated or modified by fermentation, generally with molds, but sometimes with bacteria, yeasts or mixtures of micro-organisms. Well known examples are soybean milk, soybean curd or tofu, Chinese soybean cheese, soy

sauce, miso and tempeh.

Soybeans contain 40 - 50% protein and offer an excellent source of this vital nutrient. If acceptable low-cost, high protein foods could be produced in large quantities from soybeans these could help toward alleviating the extreme protein shortage in the world to-day. The literature on the utilization of soybeans for the production of such important products as soybean milk, soybean curd, Chinese soybean cheese, soy sauce, miso, tempeh and others is reviewed below:

Soybean Milk

According to Miller (1943), the Oriental people have come to understand by experience, rather than by scientific deduction, that soybean protein is essential to livelihood and cannot, with safety, be omitted from their diet. They have learned that the greatest nutritional value from soybean is obtained through a water extraction of its protein.

Piper and Morse (1923) reported that soybean milk is prepared by thoroughly washing the soybeans and then soaking them in water for several hours. The soybeans are then ground in a stone mill and three times the volume of water added to the dense milky liquid. The mixture is then boiled and filtered, yielding soybean milk.

Smith and Beckel (1946) have stated that soybean milk, as ordinarily produced, does not have the bland flavor or smooth texture

of cow's milk.

Miller (1937) described a process in which the beany flavor was volatilized from the soybean milk by boiling. After the finely ground slurry was boiled, the solid residue was removed by centrifugation. In order to make a balanced milk, sugar, vegetable fat and salt were added. This mixture was then actively boiled with agitation for 30 - 60 minutes, and subsequently homogenized and bottled or spray-dried.

According to Piper and Morse (1923), soybean milk with some added sugar is drunk by the Chinese in the early morning and is also eaten as a thin broth and with salted pickles. Vegetable milk is extensively used for infant feeding throughout China and in many cities and towns it is produced in factories and delivered in bottles to regular customers.

Fomon (1959) has recommended soybean milk for feeding babies that are allergic to cow's milk.

Soybean milk, when inoculated with Lactobacillus acidophilus, produces an especially potent buttermilk type of product that has a characteristic flavor. The striking and important feature of the soybean milk culture is the fact that while a cow's milk culture deteriorates so rapidly that in a few weeks its potency is practically lost, the soybean milk culture may retain its vitality for many months. (The Battle Creek Food Co. 1947).

Hand et al. (1964), prepared dry soybean milk and found that this product was of superior quality than that made directly from

whole soybeans without including a water extraction step. The yield was better, and the power and labor costs were reduced. In the manufacture of dry soybean milk from whole soybeans a homogenizer is added to the processing line, but an evaporator and filter press are eliminated.

Soybean Curd and Soybean Cheese

Soybean curd or tofu is made by precipitating the protein from the soybean milk previously described by means of acids or salts. According to Piper and Morse (1923), the coagulating agents most commonly used throughout the Oriental countries are the concentrated mother liquor obtained in the manufacture of salt from sea water, burned powdered gypsum and magnesium chloride. After the curd settles, the supernatant liquor is removed and the curd is placed on cloths spread in wooden trays which serve as moulds. Sufficient water is removed by pressing until the curd is solid enough to be handled. The curd has the consistency of cream cheese and is cut into small squares and sold immediately.

Smith et al. (1960) found that tofu made in pilot-plant investigations from different varieties of United States and Japanese soybeans showed observable differences in color and texture. The yield of tofu varied with variety and location, but the average yield from U. S. soybeans was about the same as from Japanese soybeans. Original protein and oil content of the soybeans influenced the yield and the final protein and oil content of the tofu.

According to Miller et al. (1952), soybean curd of the Japanese type contains approximately 10% of a high quality vegetable protein with no crude fiber, and when precipitated with calcium chloride it is as good a source of calcium as is milk. Miller (1943) has stated that Oriental people obtain 90% and possibly even more soybean protein in the form of tofu. Fresh tofu contains 84 - 90% moisture, 5 - 8% protein, 3 - 4% fat, and about 2 - 4% carbohydrate (Piper and Morse, 1923). Because of high moisture content, tofu is subject to spoilage and is generally prepared and used each day.

Several attempts have been made to prepare ripened cheese from soybean curd. Katayama (1906) in one experiment, introduced the characteristic microbes of Swiss cheese by adding casein and Swiss cheese to pressed tofu mixed with salt. In another experiment the casein was replaced with lactose. Upon aging, cheese having an agreeable taste were obtained, but unlike Swiss cheese they contained no holes. Wai (1929) described a process for the production of Chinese soybean cheese or sufu. First, the tofu was cut into blocks, arranged on bamboo trays and left in the fermentation chamber for a month. The average temperature of the fermentation was 14°C. After this treatment, the blocks were put into large earthenware barrels and salt and Shoushing wine were added. The barrels were closed and left for about three months after which time the process was complete. The products were white in color. When a red cheese was required, an inoculum of Monascus purpureus was added to the aging-solution. The use of Monascus purpureus for this purpose was first recognized by Church (1920). Corville (1929) reported that Chinese soybean cheese was made by fermentation of tofu with a species of

Mucor sufu. According to Wai's data (1964), soybean cheese contains 73.9% moisture, 10.36% water insoluble protein, 1.26% water soluble nitrogen compounds and 4.3% lipid.

Smith (1949) stated that variations in Chinese soybean cheese can be caused not only by the type of micro-organism used but also by the proportion of salt and type of solution in which the cheese is aged. The cheese appears to vary somewhat with the locality in which it is produced, a variation probably caused by the influence of climatic conditions on the activity of the fermenting micro-organisms.

Different species of fungi isolated from Chinese soybean cheese have been reported by Hesseltine (1965). They all belong to the family Mucoraceae. The role of fungi is to excrete enzymes that breakdown the soybean protein to peptides and amino acids. Hesseltine also stated that the mold used should be dense and thick enough to form a solid film over the surface of the tofu. The mold should also not impart a disagreeable odor or taste.

Soy Sauce

Soy sauce is a dark brown salty liquid made by the fermentation of soybeans and additional starchy components. It is used in the preparation of food and also as a table condiment. According to Minor (1945), soy sauce consists of a mixture of amino acids, peptides, polypeptides, peptones, simple proteins, purines and lesser amounts of other organic compounds suspended in an 18% salt solution. Total solids constitute about 25% of the unmodified product.

There are two methods by which soy sauce is commonly made. One is the ancient Chinese method of proteolytic enzyme digestion and the other is the acid hydrolysis of certain protein-bearing cereal grains. In both methods the starting material, containing the necessary protein, consists mainly of soybeans and wheat.

Wang and Ni (1936) stated that the time required to carry out fermentation by the original Chinese method is about one year. Of the many methods developed to shorten the process they believe the Togano and Kwantou processes deserve special attention. The Togano process involves decomposition of soybean protein separately from the carbohydrate fermentation with wheat. Koji (mold fermented rice) is mixed with salt solution and allowed to stand with constant stirring at 42°C., the decomposition being complete in about twenty days. Carbohydrate in the form of partially fermented wheat is added to the koji-salt mixture and the fermentation allowed to continue for two more months. Wang and Ni, however, claimed that soy sauce produced by this method has an inferior flavor to that produced by the Kwantou process. An investigation of the latter process included an analysis of the raw material, intermediate and final products. The process requires about three months and the superior flavor obtained was attributed to washing the mold mycelia from the culture after twelve days, followed by a second fermentation.

Minor (1945) has described the preparation of soy sauce by the use of specific types of molds such as Aspergillus flavus, A. niger, and A. oryzae, each mold imparting a distinctive flavor and character to the finished product. Two parts of precooked soybeans

are mixed with 1.5 parts crushed roasted wheat. The entire mass is inoculated with a mold culture such as A. oryzae. When the mold has developed sufficiently the entire mass is placed in a salt solution and allowed to react in sterilized glass vessels at 120°F for six to eight weeks. The sauce is then pressed out of the grain mass, filtered, pasteurized and bottled.

Church (1923) pointed out that, in addition to changes brought out by molds, bacteria and yeasts are also active during fermentation. Lockwood (1947) developed a process in which suitable strains of A. oryzae, Zygosaccharomyces soyae and Lactobacillus delbrueckii were used under controlled conditions for the production of soy sauce on a factory scale.

An alternate method of producing soy sauce is acid hydrolysis of soybeans. According to Minor (1945), a mixture of soybean meal and wheat proteins is hydrolyzed by refluxing with constant boiling hydrochloric acid (20% solution) until a maximum concentration of amino acid nitrogen has been obtained. After the reaction end-point has been reached, the batch is neutralized with a 50% solution of sodium hydroxide to pH 4 - 5. The sauce is then ready to be placed in hardwood storage tanks for aging prior to bottling. Minor also stated that soy sauce obtained from the acid hydrolysis method contained at least five times as much amino acids as sauce prepared by the mold enzyme method. However, valuable food components produced by the mold enzyme method are destroyed by acid hydrolysis. Acid hydrolysis destroys tryptophan, an essential amino acid, as well

as other basic amino acids of biological importance.

According to Yokotsuka (1960), the ratio of amino nitrogen to total nitrogen has been considered as a criterion for judging the quality of soy sauce, with a high ratio indicating high quality. Usually the amino nitrogen content is about 40 to 50% of the total nitrogen.

Miso

Miso is a fermented food made from soybeans and rice with the addition of salt. It is widely used in Japan as a base for a hot breakfast soup. It is also used as a flavoring agent with various foods, including vegetables and meats. There are many different kinds of miso which are distinguished by color, taste and keeping quality; the differences consisting chiefly in the amount of salt added, the amount of koji used and the time and temperature of fermentation. Shibasaki and Hesseltine (1962) reported that white miso contains more rice than soybeans and contains 4 - 8% salt; brown miso contains 50 - 90% soybeans and 11 - 13% salt. Moisture content, usually 48 - 52%, greatly affects the rate of fermentation.

According to Shibasaki and Hesseltine (1961), the manufacture of miso is a process involving two separate and distinct fermentations. The first involves the aerobic pure culture fermentation of rice with selected strains of A. oryzae to prepare koji as a source of enzymes and nutrients for the second fermentation - the fermentation of koji, salt and soybeans. In the second fermentation the inoculum used is

a suitable sample of good miso from an earlier fermentation. Such a mixed inoculum has obvious disadvantages. Contaminating micro-organisms are carried from one fermentation to the next. Secondly, since most types of miso require months to ferment, followed by an aging period, it is apparent that the proper micro-organisms for the fermentation become reduced in number and vigor; hence, a longer period is required for them to initiate active growth and multiplication.

Hesseltine and Shibasaki (1961) have developed a new process of manufacturing miso in which a pure culture of Saccharomyces rouxii NRRL Y-2547 is used as a starter. According to their results, a pure culture fermentation eliminates all the contaminating micro-organisms which are introduced by using old miso as a starter. Another advantage they noted was that the fermentation as judged by odor always began much sooner in the pure culture fermentation than in the old method.

Changes occurring during miso fermentation have been reported by Shibasaki and Hesseltine (1962). During the fermentation the rice starch is digested by amylase and maltase to dextrin, maltose and glucose. Soybean protein is digested by proteolytic enzymes to peptides and amino acids. The main soybean protein is glycinin (80 - 90%) which contains almost 20% glutamic acid. The true miso flavor is thought to be entirely contributed by soybeans while the rice contributes sweetness. The soybean lipids present are attacked by microbial lipases to a limited extent yielding fatty acids. Many lactic acid bacteria develop in koji and form considerable

amounts of lactic acid. This acid gives the sour taste to miso and assists the salt in preventing spoilage. During fermentation, some yeasts produce ethyl alcohol, higher alcohol and succinic acid. The alcohol together with esters formed by combination with fatty acids contribute to the pleasant odor of miso. Some pigments are produced by the browning reaction of amino acid with sugar. This is the so-called soyamelanine and in part gives the color to miso. Such chemical reactions are expected to continue during aging. The fermentation of white miso requires but a few weeks for completion, while that of the brown miso requires months.

Tempeh

Tempeh is a popular Indonesian food produced by fermenting soybeans with a species of Rhizopus. It is high in protein and unsaturated oil; when fried in oil, it has a pleasing flavor and texture.

Autret and van Veen (1955) have suggested tempeh as a low-cost product for use in worldwide food programs.

van Veen and Shaefer (1950) have reviewed the literature and described the production of tempeh. Their studies suggest that tempeh is more easily digested than the original soybean.

Tempeh is made in a primitive fashion in Indonesia by wrapping dehulled and cooked soybeans in banana leaves, inoculating with a mixed culture of Rhizopus and other micro-organisms, and allowing them to ferment for about two days. Methods have been

devised by Martinelli and Hesseltine (1964) to make tempeh rapidly in large amounts by pure-culture fermentations in shallow wooden and metal trays with perforated bottoms and covers. Excellent tempeh has also been made in perforated plastic bags and tubes. The latter containers are specially good because soybeans can be fermented in a package. All fermentations are completed in 24 hr at 31°C.

Steinkraus et al. (1960) have developed a process in which a pure culture of mold, Rhizopus oryzae, is used under controlled conditions for the production of tempeh. The soybeans are soaked overnight in dilute lactic acid solution at room temperature. The hydrated soybeans are drained and the acidified soak water is saved. The soybeans are skinned and placed in stainless-steel pans and covered with the acidified soak water and cooked at 100°C for 90 minutes. The soybeans are then drained, cooled to 37°C, inoculated with R. oryzae, then incubated at 37°C. During the fermentation, the soybeans become bound together into a compact cake by mold mycelia. The soybean cakes are then sliced and dried or roasted, cooked in soup or deep fried in fat before they are eaten.

The principal organism involved in the tempeh fermentation, according to van Veen and Shaefer (1950), is R. oryzae. Although they found numerous bacteria and yeasts present, they concluded that they were not of considerable importance to the process. Steinkraus et al. (1960) reported that the bacteria were definitely undesirable, contributing off-flavors to the tempeh if they were allowed to develop. If the pH of the soybeans was adjusted to 5 or below, bacteria did not develop on the soybeans.

Changes occurring in the soybeans during the tempeh fermentation have been investigated by Steinkraus et al. (1960). The temperature of the fermenting bean mass was indicative of the relative rate of mold growth. For the first few hours after inoculation, there was a lag during which germination of spores took place, following by several hours of slow mold growth. When mold growth became rapid, the temperature of the bean mass rose above that of the incubator. After 4 - 5 hr of accelerated mold growth, the temperature reached 43 - 44°C after which it gradually fell as mold growth subsided. At this stage, the beans were bound into a compact mass by the mold mycelia. Following rapid mold growth, sporulation and ammonia production occurred due to protein breakdown. During the period of most rapid mold growth, the soluble solids increased from 13% to 21%, and as a result of further enzyme digestion, the soluble solids continued to rise to 27.5%. The soluble nitrogen rose from 0.5% to nearly 2.0% while the total nitrogen remained relatively constant, about 7.5%. The pH increased from an initial level of 5.0 to almost 7.6, changing from 6.0 to 6.7 during the period of most rapid mold growth. The tempeh was at its optimum quality when the pH was in the range of 6.3 to 6.5. Reducing substances showed an overall decline during the fermentation. The fiber content increased from 3.7% in the skinned soybeans to 5.85% in the tempeh due to the development of mold mycelia.

Changes in soybean lipids during the tempeh fermentation have been studied by Wagenknecht et al. (1961). Total fat (as ether soluble solids) remained fairly constant. Roughly one-third of

the neutral fat of the soybeans was hydrolyzed by the fungal lipase. Of the free fatty acids liberated, only linolenic acid was utilized by the mold. Gyoygy (1961) reported that the nutritive value of one lot of freeze-dried tempeh prepared from Seneca soybeans was equivalent to that of skimmilk and much higher than the unfermented soybean control. Steinkraus et al. (1961) stated that the nutritive value of tempeh decreased with increased fermentation time. Changes in some B vitamins during molding of soybeans by R. oryzae in the production of tempeh have been reported by Roelofsen and Talens (1964). Of the thiamine present in the cooked cotyledons, about one-third was used up by the fungus. Riboflavin and niacin, however, increased considerably.

According to Smith et al. (1964), loss of solids and protein in dehulling, soaking, washing, and cooking of soybeans before fermentation does not reduce the nutritive value of either cotyledons or full-fat grits used to make tempeh. Since pancreatic hypertrophy does not occur in rats fed tempeh, the heat used in normal preparation of tempeh is sufficient to destroy the factors in raw soybeans responsible for poor growth and pancreatic hypertrophy. Methionine supplementation of tempeh significantly increases rate of rat growth.

Steinkraus et al. (1965) have developed a pilot-plant process for the production of dehydrated tempeh. The process involves size grading, dry dehulling, hydration and cooking in boiling dilute acid solution, draining, cooling, inoculation with the tempeh fungus, fermentation for 18 hr at 35 - 38°C and

75 - 85% relative humidity, dehydration and packaging in moisture-proof plastic bags.

Miscellaneous Products

In addition to the products described, whole soybeans are also used for making sprouts, yuba and other fermented food products such as natto and hamannato. How these Oriental food products are prepared from soybeans has been described by Smith (1949, 1958).

Djien and Hesseltine (1961) have described a process used for making ketjap, an Indonesian soy sauce made from black soybeans.

According to Smith and Wolf (1961) the starting material for most soybean foods used in the United States is dehulled, defatted and substantially undenatured soybean meal containing 50% protein or more. Made from specially prepared meal are food-grade soybean protein isolates and concentrates such as soy flour, acid-precipitated protein, soluble soybean proteinate, 70% protein concentrate, Gelsoy-type products and dry soybean whey solids. These products are not used alone but are added to other foods for functional effects or as a nutritional supplement.

Use of enzymatically degraded soybean protein for production of alcoholic beverage has been investigated by Iida and Sakamoto (1963). The purpose of using degraded soybean protein is to enhance the flavor of the alcoholic beverage. The proteolytic enzymes of A. oryzae, Bacillus subtilis or Streptomyces griseus are used for digesting soybean protein.

EXPERIMENTAL

Preparation of Soybean Milk

450 g of dry mature soybeans (Grade No. 1, W.G. Thompson & Son,Limited, Blenheim, Ontario, Canada) were washed and soaked in 1400 ml of tap water in a cooler (5°C) overnight. The next day the water was discarded, the soaked soybeans (Plate 1) were put into a Waring blendor and tap water was added in the ratio of 9 to 1. The mixture was blended for 2 minutes, the extract filtered through linen and the residue discarded. The resulting liquid was autoclaved for 20 minutes at 15 pounds pressure (121°C) and used for the preparation of starter and soybean curd. The heat treatment results in the removal of the beany flavor, sterilization of the soybean milk and destruction of anti-digestive factors present in raw soybeans.

Micro-organisms Used for Fermentation

Several freeze-dried cultures of lactic starters, Dagano cheese culture, Streptococcus diacetilactic and Streptococcus thermophilus were used in the earlier investigations. Only Str. thermophilus developed sufficient acid to coagulate the soybean milk within a period of five hours. As a result, Streptococcus thermophilus 101 (Klenzade Products, Division of Economics Laboratory, Inc., Beloit, Wisconsin, U. S. A.) was used in the present study.

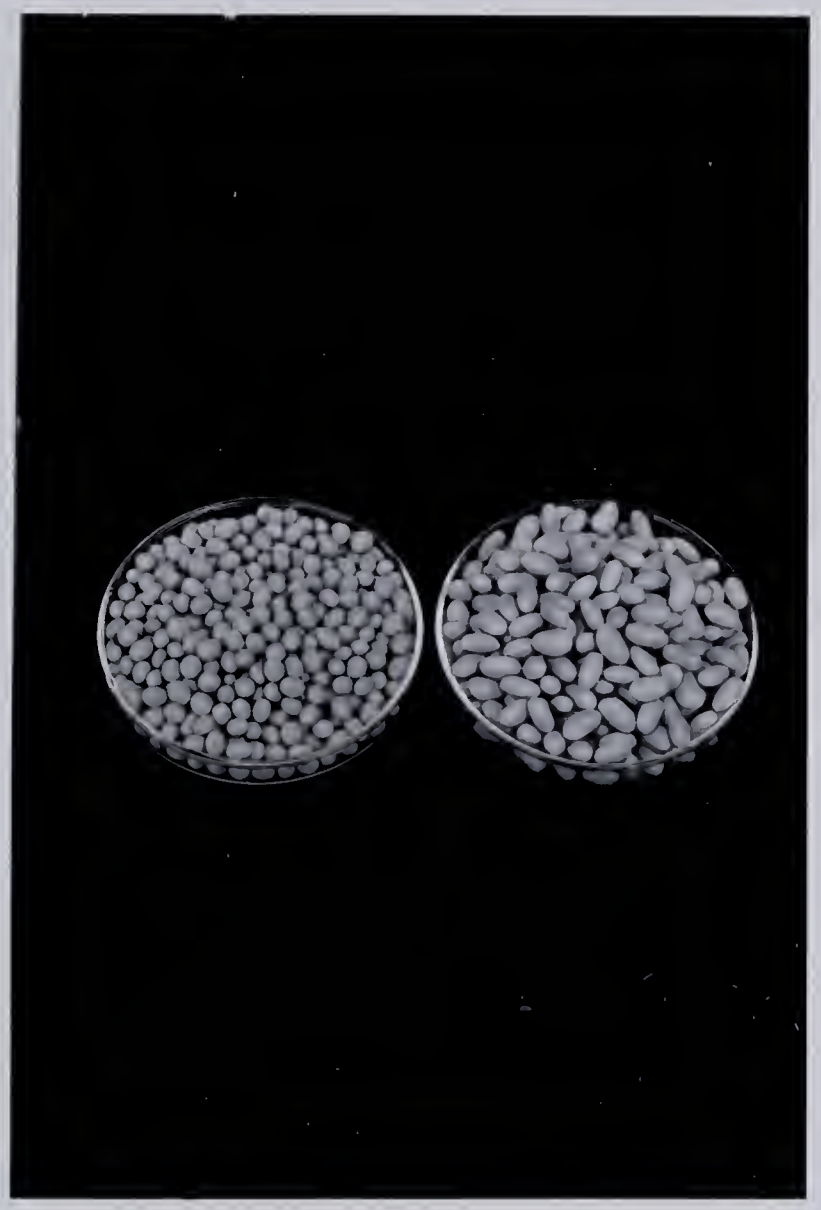


Plate 1. Dry soybeans (left) and soaked soybeans (right)

Development of Starter

200 mg of freeze-dried culture was added to a flask containing 200 g of autoclaved soybean milk and the mixture incubated at 32°C for 15 hours. One ml of this mother starter was then added to 200 g of autoclaved soybean milk and after incubation the process was repeated. The resulting starter culture was stored in the cooler until required.

Preparation of Soybean Curd

Autoclaved soybean milk was converted into soybean curd by three different methods as follows:

(a) Addition of calcium sulfate

3 kg of soybean milk were cooled to 65°C and, while stirring slowly, the curd was precipitated by adding 9 g of calcium sulfate suspended in 72 ml of distilled water (Smith *et al.*, 1960). Stirring was stopped as soon as the calcium sulfate was added to avoid breaking the curd. The curd was then transferred to a round stainless steel hoop (Plate 2) and pressed for 21 hours under a pressure of twenty pounds.

(b) Addition of acetic acid

The procedure used was similar to that of Method (a), the only difference being that sufficient 4% acetic acid was mixed with the soybean milk to lower the pH to 4.5 to cause the curd to separate from the whey (Hand *et al.*, 1964).

Plate 2. Hoop (12 1/2 cm x 14 1/2 cm) used in the manufacture of soybean cheese.

(c) Lactic acid fermentation

3 kg of autoclaved soybean milk were cooled to about 41°C and 5% of starter culture was added. The mixture was incubated at 41°C in a water bath. When fine lines of whey appeared where a knife had cut the jelly-like curd, the curd was ready to be cut. After cutting, the curd was cooked by raising the temperature of the water bath to 48°C. When cooking finished, the curd was put into the hoop and then pressed for 21 hours.

Additives

Several milk-clotting enzymes such as rennin, pepsin and papain were used in earlier investigations to determine their ability to coagulate the soybean milk. However, none of them possessed soybean milk-clotting activity. In an attempt to minimize the time from the addition of starter to the time the curd was ready to be cut, rennet extract and skimmilk were added to the soybean milk.

Cheese Prepared

A total of twelve cheese were prepared. Two cheese were made by each of the three methods mentioned above. The purpose of this experiment was to compare yield of protein, moisture content, protein content and hardness of soybean cheese using starter organisms with conventional methods. In addition, six cheese were made according to the following scheme:

Cheese No.	Starter	Rennet extract*	Skimmilk+
1	X		
2	X		
3	X	X	
4	X	X	
5	X	X	X
6	X	X	X

* one ml of commercial rennet extract diluted to 20 ml with sterile distilled water

\+ 15% of weight of soybean milk

The latter six cheese were salted by a dry-salting method; one and one-half percent of salt on the basis of weight of soybean cheese were used. The cheese were then waxed and ripened at 20°C for 63 days. The purpose of this experiment was to study the shelf life, palatability, chemical, physical and microbiological aspects of cheese prepared by three different methods. Plate 3 shows a typical soybean cheese.

Sampling

The technique for sampling the kettle contents for bacterial investigations and pH determinations during manufacturing process was that developed by Frazier et al. (1934). A sterile 50-ml beaker was used for sampling. The curd was allowed to settle, the whey was poured off into a sterile beaker and the curd transferred to a sterile mortar and ground for one minute. Sodium citrate was then added at the rate of 0.2 gram for each gram of curd and the grinding continued

Plate 3. Typical soybean cheese.

until a smooth, even paste was formed. The decanted whey was then poured in and the contents of the mortar were thoroughly stirred until the resulting fluid was homogeneous. One ml of this fluid was used for making plate counts.

For making pH determinations the samples were taken in the same manner; curd and whey were separated, and the curd was ground without addition of sodium citrate. The decanted whey was returned to the curd and pH determinations were made on the mixture of whey and curd.

During the ripening process, samples were removed at weekly intervals and pH, moisture, total and water-soluble nitrogen and bacterial numbers were determined. Samples used for moisture, nitrogen and bacterial count determinations were obtained by removing a 1 1/2 inch plug, using a sterile trier, from the hoop side of the soybean cheese half-way between the top and bottom surfaces. 1/2 inch of the plug was cut off, measuring from the hoop end of the plug. The remainder was cut into two halves longitudinally, one part being used for moisture determination and the other for bacterial counts. The dried sample resulting from moisture determination was used for total and water-soluble nitrogen determinations.

Determination of Bacterial Numbers

The medium used for the enumeration of bacteria in soybean cheese during the manufacturing and ripening processes had the following composition:

Medium*

Agar	15 g
Bactotryptone	5 g
Yeast extract	3 g
Lactose	1 g
Bromo-cresol purple powder	0.05 g
Phenol red powder	0.12 g
Distilled water	1000 ml
Final pH	6.8

The sample of cheese used for bacterial counts during ripening was ground in a sterile mortar with sodium citrate and distilled water, by the method described by Burkey (1931)

pH Measurement

For the measurement of the pH the glass electrode of a Beckman Model 72 pH meter was inserted into a depression made in the side of a 5/8 inch diameter plug of soybean cheese.

Moisture Content

Moisture content was determined by drying samples to constant weight in a Majonnier apparatus at 100°C.

Total and Water-Soluble Nitrogen Content

Total nitrogen was determined by standard micro-Kjeldahl procedure. Water-soluble nitrogen was determined by adaptation of the method of Smith et al. (1952). A finely ground sample was

* A modification of the medium developed by Donovan and Vincent, J. Dairy Research, 22, p. 43. (1955).

extracted with water in a mechanical shaker at 60 rpm (Eberbach Corporation, Ann Arbor, Michigan, U. S. A.) for two hours. The suspension was centrifuged for 30 minutes at 10,000 rpm. The supernatant was then filtered through a Whatman No. 1 filter paper and the filtrate analyzed by micro-Kjeldahl procedure for water-soluble nitrogen.

Determination of Hardness

Hardness of soybean curd or soybean cheese was measured by means of a "Precision" penetrometer (Precision Scientific Co., Chicago, U. S. A.) [Plate 4] and shear press (L.E.E. Incorporated, Washington, 1, D.C., U. S. A.) [Plate 5]

Plate 4. "Precision" penetrometer used to measure the hardness of soybean curd.

Plate 5. Shear press used to measure the hardness of soybean curd and ripened soybean cheese.

RESULTS

Characteristics of Soybean Cheese Prepared Using Acetic Acid, Calcium Sulfate and Starter Organisms

Yield of precipitated protein, protein content, moisture content and hardness of soybean cheese resulting from three different methods of preparation are shown in Table 1.

Yield of precipitated protein

Yield of precipitated protein was calculated by substraction of the nitrogen in the supernatant liquid from total nitrogen content of the soybean milk. The protein conversion factor used was 6.25. The yield of precipitated protein by three methods of preparation is illustrated in Figure 1. The yield of precipitated protein as prepared by lowering the pH of soybean milk to 4.5 with acetic acid was the highest of the three methods used, while lactic acid fermentation gave a slightly better yield of protein than did addition of calcium sulfate.

Moisture content

It will be noted that moisture content of soybean curd obtained by lactic acid fermentation is the lowest of the three methods of preparation. It is likely that acid produced by starter organisms during fermentation may facilitate drainage of whey from the interior of the soybean curd.

Protein content

The protein content of soybean curd is the highest when

Table 1. Yield of precipitated protein, moisture content, protein content and hardness of soybean curd obtained by three different methods of preparation.+

Method of precipitation	Yield of precipitated protein %	Moisture content %	Protein content %	Hardness	
				Penetrometer*	Shear press**
Addition of acetic acid	67.8	77.6	56.3	126	23
Addition of calcium sulfate	54.1	84.8	51.3	141	7
Lactic acid fermentation	55.0	76.9	52.4	82	42

* The higher the value the softer the soybean curd

** Value is expressed as pounds per 100 grams of sample measured

+ All values are the means of two determinations.

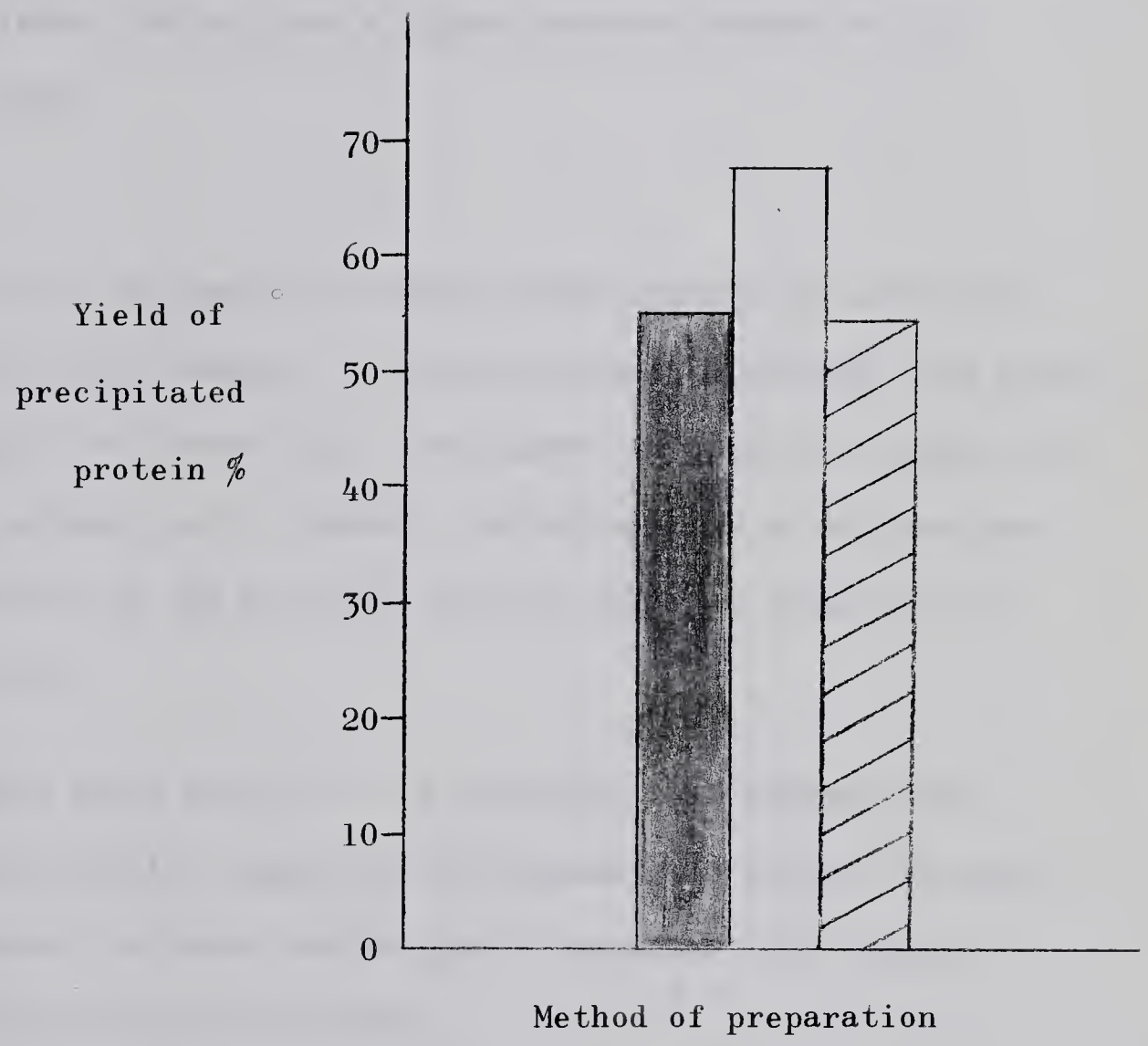


Figure 1. Yield of precipitated protein obtained by three different methods of preparation of soybean curd

acetic acid was used for precipitation, while that of curd prepared by lactic acid fermentation is slightly higher than that of curd obtained by the addition of calcium sulfate. Lowering the pH of soybean milk to the isoelectric point of soybean protein appears to be a major factor which gives a higher protein content of the finished product.

Hardness

It can be seen that soybean curd prepared by lactic acid fermentation is the hardest. Moisture content of soybean curd appears to be an important factor since the higher the moisture content the softer the soybean curd. However, the differences in hardness may also be affected by the different physico-chemical properties of soybean protein.

From these results it is concluded that soybean curd prepared using starter organisms is comparable in protein recovery, protein content, moisture content and is superior in hardness to those resulting from other methods.

Characteristics of Soybean Cheese Prepared Using Starter Organisms, Rennet Extract and Skimmilk

Manufacturing data

The make records of soybean cheese using soybean milk and starter only are shown in Table 2 and Table 3. It will be noted that the time from the addition of starter to the time of cutting is three hours and ten minutes.

Table 2. Manufacturing record of soybean cheese (soybean milk plus lactic starter)

Amount of soybean milk	3 kg
pH of soybean milk	6.60
Amount of starter used	5%
pH of starter	4.69

Manufacturing steps	Cheese No. 1			
	Time Hr Min.	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Starter added to soybean milk	0	41	6.44	19
Curd cut	3:10	41		
Cooking begun	3:15	41	5.41	88
Cooking finished	3:45	48		
Dipping	3:50		5.26	120
Pressing	4:05			
Yield of soybean cheese	662 g			

Table 3. Manufacturing record of soybean cheese (soybean milk plus lactic starter)

Amount of soybean milk	3 kg
pH of soybean milk	6.61
Amount of starter used	5%
pH of starter	4.80

Manufacturing steps	Cheese No. 2			
	Time Hr. Min	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Starter added to soybean milk	0	41	6.49	17
Curd cut	3:10	41		
Cooking begun	3:15	41	5.43	83
Cooking finished	3:45	47.9		
Dipping	3:50		5.30	100
Pressing	4:05			
Yield of soybean cheese		649 g		

Table 4. Manufacturing record of soybean cheese (soybean milk plus lactic starter plus rennet extract)

Amount of soybean milk	3 kg
pH of soybean milk	6.59
Amount of starter used	5%
pH of starter	4.70

Manufacturing steps	Cheese No. 3			
	Time Hr Min	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Starter and rennet added to soybean milk	0	41	6.43	17
Curd cut	3:10	41		
Cooking begun	3:15	41	5.44	84
Cooking finished	3:45	48		
Dipping	3:50		5.28	110
Pressing	4:05			
Yield of soybean cheese		615 g		

Table 5. Manufacturing record of soybean cheese (soybean milk plus lactic starter plus rennet extract)

Amount of soybean milk	3 kg
pH of soybean milk	6.60
Amount of starter used	5%
pH of starter	4.62

Manufacturing steps	Cheese No. 4			
	Time Hr Min	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Starter and rennet added to soybean milk	0	41	6.42	21
Curd cut	3:10	41		
Cooking begun	3:15	41	5.40	100
Cooking finished	3:45	48.1		
Dipping	3:50		5.24	140
Pressing	4:05			
Yield of soybean cheese		630 g		

Table 6. Manufacturing record of soybean cheese (soybean milk plus lactic starter plus rennet extract plus skimmilk)

Amount of soybean milk	3 kg
pH of soybean milk	6.60
Amount of starter used	5%
pH of starter	4.74

Manufacturing steps	Cheese No. 5			
	Time Hr Min	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Skimmilk, starter and rennet added to soybean milk	0	41	6.49	18
Curd cut	2:30	41		
Cooking begun	2:35	41	5.47	79
Cooking finished	3:05	48		
Dipping	3:10		5.24	110
Pressing	3:25			
Yield of soybean cheese		596 g		

Table 7. Manufacturing record of soybean cheese (soybean milk plus lactic starter plus rennet extract plus skimmilk)

Amount of soybean milk	3 kg
pH of soybean milk	6.59
Amount of starter used	5%
pH of starter	4.61

Manufacturing steps	Cheese No. 6			
	Time Hr Min	Temp. °C	pH of soybean milk or curd plus whey	Bacterial counts x 10^6 per ml of sample
Skimmilk, starter and rennet added to soybean milk	0	41	6.45	20
Curd cut	2:30	41		
Cooking begun	2:35	41	5.45	86
Cooking finished	3:05	48.1		
Dipping	3:10		5.21	150
Pressing	3:25			
Yield of soybean cheese	609 g			

The make records of soybean cheese using soybean milk, starter and rennet extract are shown in Table 4 and Table 5. It can be seen that the time of coagulation is not reduced by the addition of rennet extract.

The make records of soybean cheese using soybean milk, starter, rennet extract and skimmilk are shown in Table 6 and Table 7. The incorporation of skimmilk results in the reduction of the time from the addition of starter to the cutting of the curd from three hours and ten minutes to two hours and thirty minutes.

Bacterial numbers during manufacture

Plate counts were made at the time of addition of starter, at the start of cooking and immediately before dipping. The results are shown graphically in Figure 2. Each curve represents the mean of data from two soybean cheese made under similar conditions. It will be noted that starter bacteria increase in number from the time they are added to the soybean milk. The increase is likely to be more rapid in the period from the start of cooking to dipping, because the higher temperature is favorable for the growth of Str. thermophilus. The bacteria in the cheese containing skimmilk would appear to multiply at a slightly more rapid rate than those in the other cheese. This could possibly be attributed to the presence of stimulating substances in the skimmilk.

pH during manufacture

pH determinations were made at the time of addition of starter, at the start of cooking and immediately before dipping.

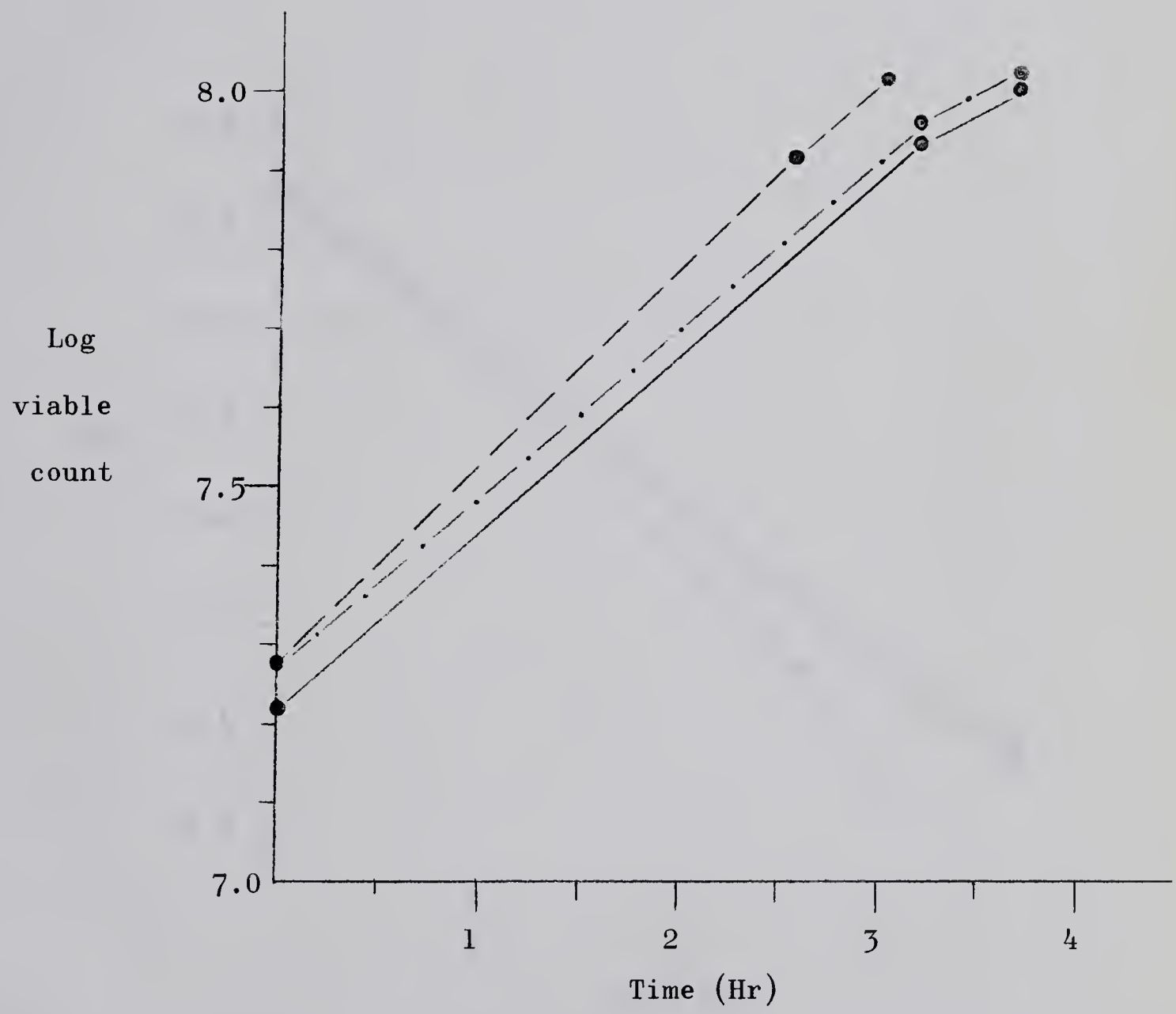
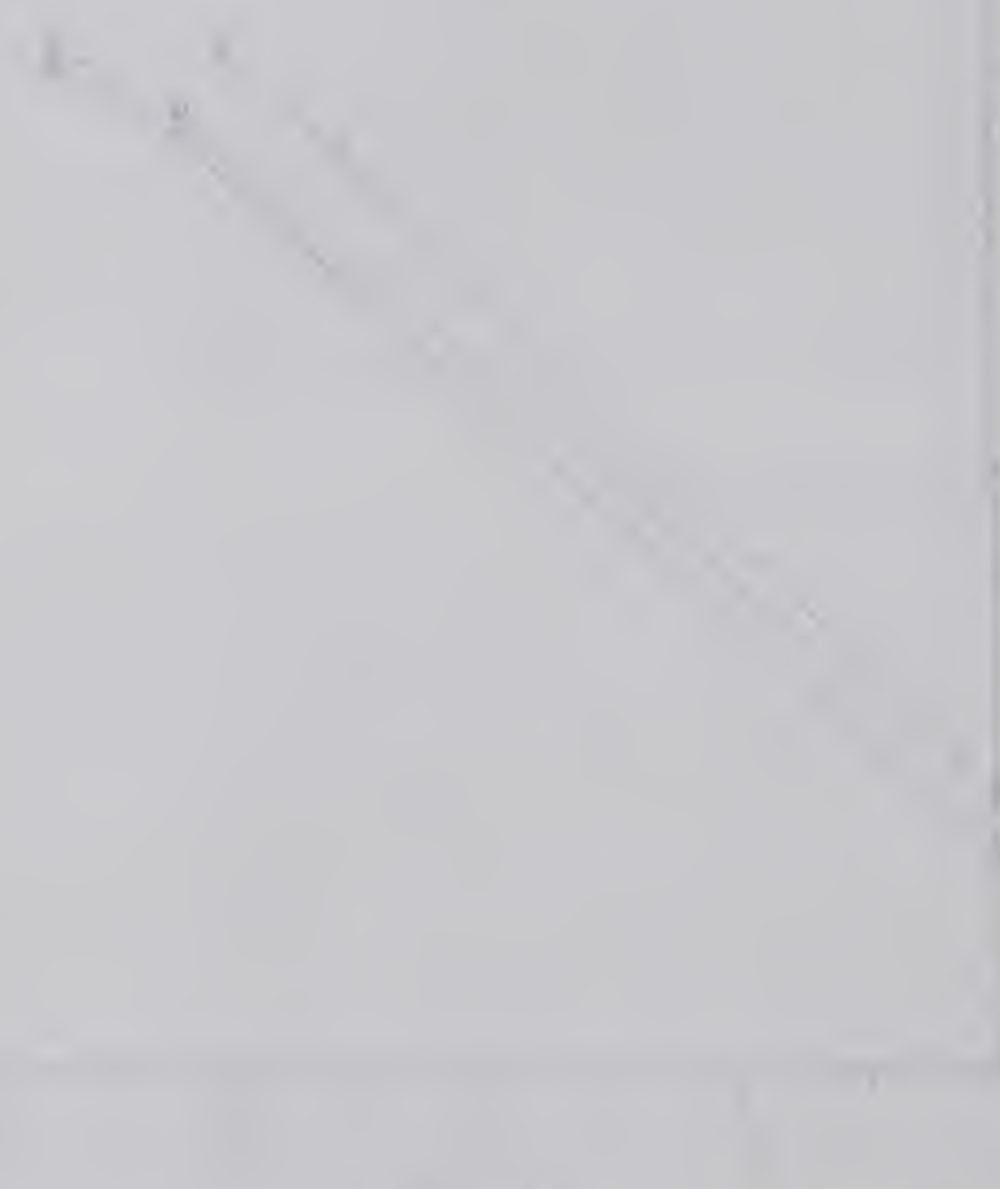


Figure 2. Bacterial changes in the manufacturing process of soybean cheese

———————— Mean of data from cheese 1 and 2 (starter only)

———.———.——— Mean of data from cheese 3 and 4 (starter and rennet extract)

—— —— —— Mean of data from cheese 5 and 6 (starter and rennet extract plus skimmilk)



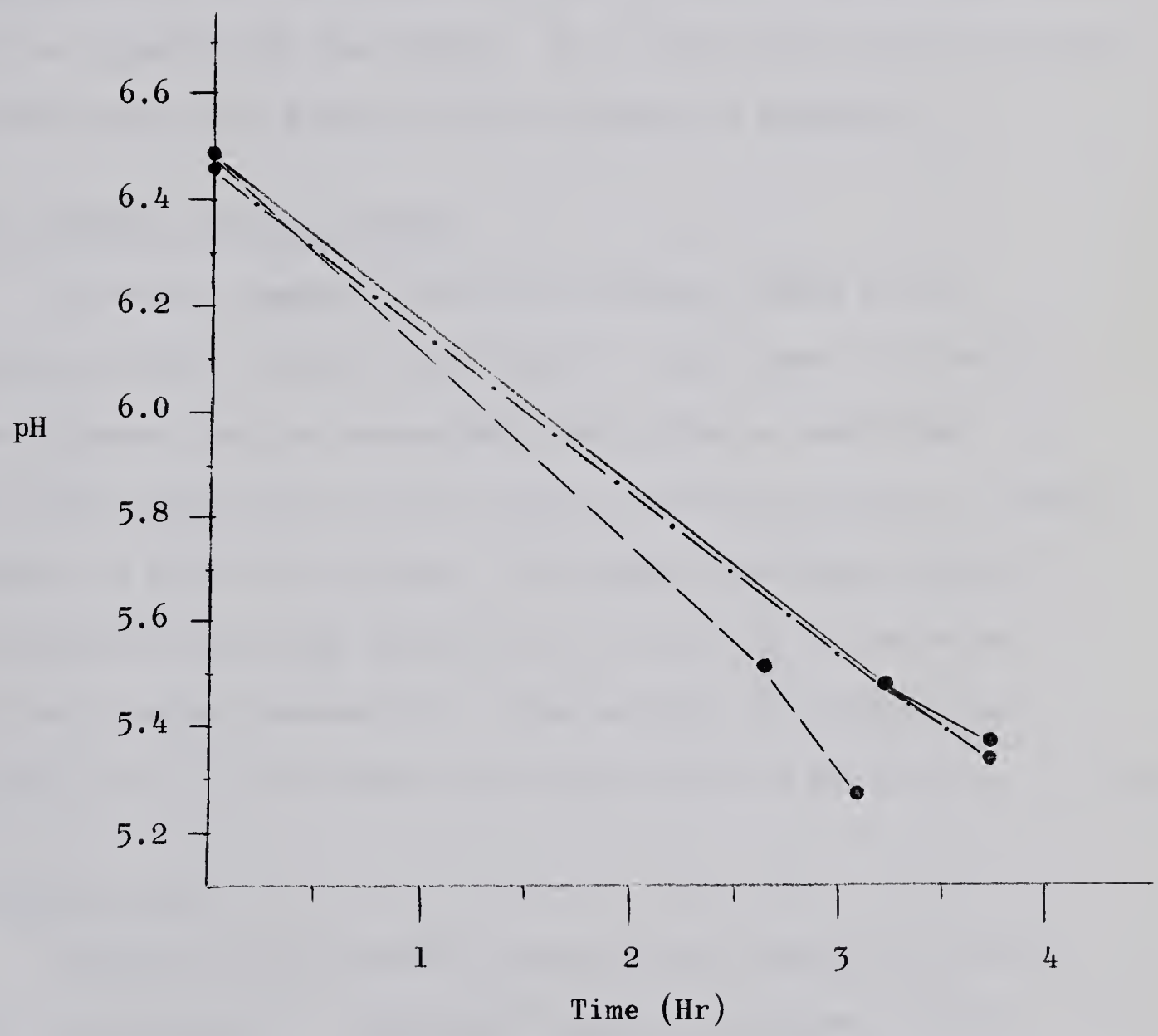


Figure 3. Acid production in the manufacturing process of soybean cheese

———————— Mean of data from cheese 1 and 2 (starter only)

——.——.—— Mean of data from cheese 3 and 4 (starter and rennet extract)

—— —— —— Mean of data from cheese 5 and 6 (starter and rennet extract plus skimmilk)

The results are shown graphically in Figure 3. Each curve represents the mean of data from two cheese made under similar conditions. It will be seen that after starter bacteria are added there is a slow and gradual decrease in pH. The decrease in pH in the cheese containing skimmilk is the most rapid and the time of incubation required is shorter as compared with the others. It is likely that the accelerated acid production is the result of the inclusion of skimmilk.

Bacterial numbers during ripening

Bacterial changes occurring in soybean cheese during ripening are shown in Table 8 and Figure 4. Each curve represents the mean of data from two cheese made under similar conditions. It will be noted that starter bacteria show a definite decrease in numbers throughout the period of ripening. The numbers decreased rapidly in the first week of ripening, probably due to salting of the cheese and the low ripening temperature. Subsequently, the decrease was more gradual and by seven weeks micro-organisms were absent from 1 g samples.

pH during ripening

Changes in pH of soybean cheese during ripening are shown in Table 9 and Figure 5. Each curve represents the mean of data from two cheese made under similar conditions. The pH values of all cheese examined show a definite decrease during the first week after manufacturing followed by a slow and gradual increase. However, in all cases the overall change in pH was very small.

Moisture content during ripening

Changes in moisture content of soybean cheese during ripening are shown in Table 10. It will be noted that there is a slow

Table 8. Bacterial changes in soybean cheese during ripening

Cheese No.	Viable count of bacteria per gram on day:									
	1	7	14	21	28	35	42	49	56	63
1	$86x10^7$	$12x10^5$	$62x10^4$	$18x10^4$	$31x10^3$	2400	34	0	0	0
2	$82x10^7$	$10x10^5$	$46x10^4$	$15x10^4$	$14x10^3$	1200	21	0	0	0
3	$98x10^7$	$80x10^5$	$90x10^4$	$20x10^4$	$32x10^3$	2200	29	0	0	0
4	$210x10^7$	$99x10^5$	$94x10^4$	$40x10^4$	$38x10^3$	7600	201	0	0	0
5	$140x10^7$	$86x10^5$	$58x10^4$	$46x10^4$	$24x10^3$	2400	42	0	0	0
6	$270x10^7$	$120x10^5$	$97x10^4$	$82x10^4$	$63x10^3$	9200	289	0	0	0

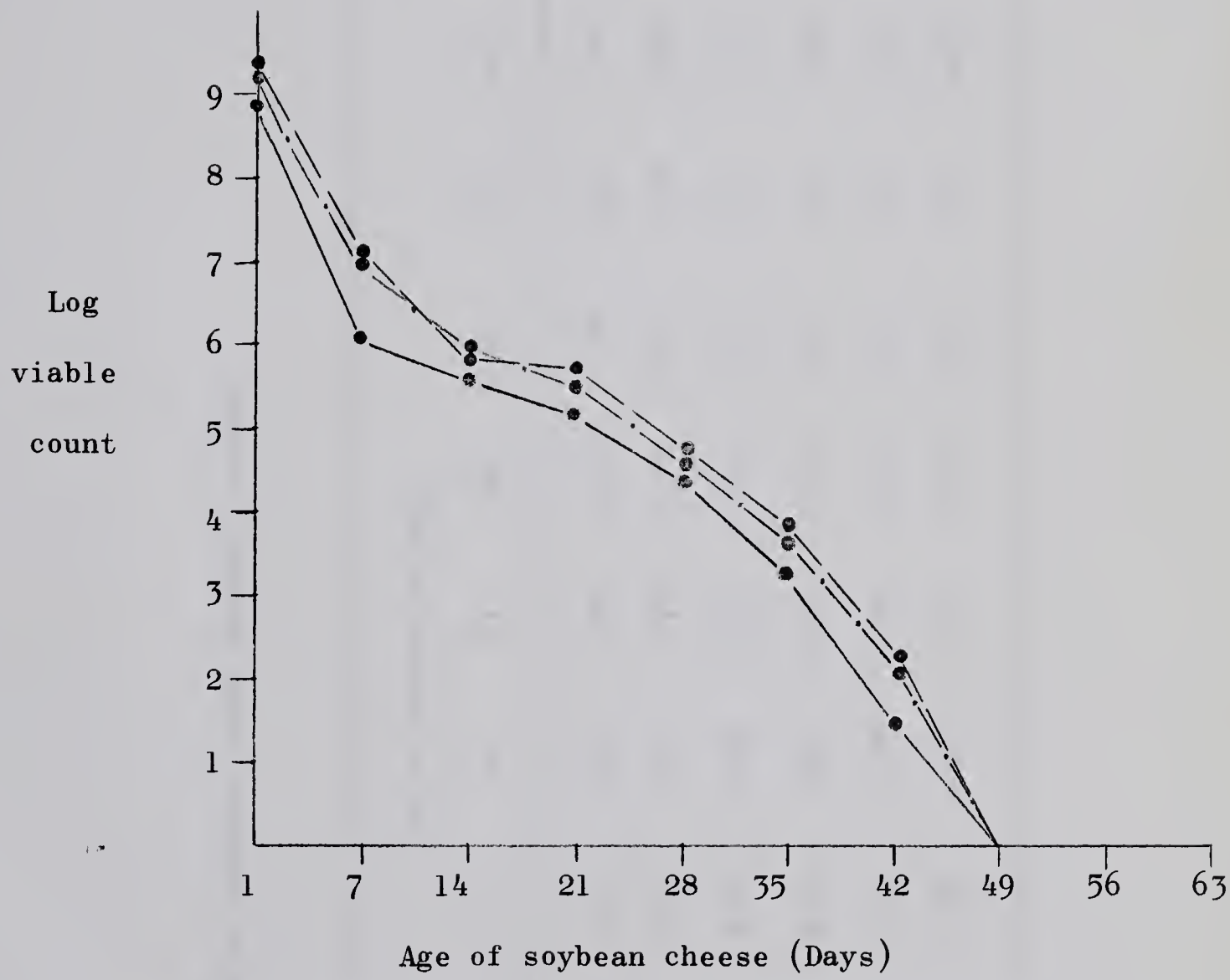


Figure 4. Bacterial changes in soybean cheese during ripening

———————— Mean of data from cheese 1 and 2 (starter only)

——— . ——— . ——— Mean of data from cheese 3 and 4 (starter and rennet extract)

——— ——— ——— Mean of data from cheese 5 and 6 (starter and rennet extract plus skimmilk)

Table 9. Changes in pH of soybean cheese during ripening.

Cheese No.	pH of cheese on day:									
	1	7	14	21	28	35	42	49	56	63
1	4.77	4.74	4.75	4.75	4.76	4.76	4.77	4.78	4.78	4.79
2	4.79	4.77	4.77	4.78	4.79	4.79	4.79	4.80	4.81	4.81
3	4.71	4.69	4.72	4.73	4.74	4.74	4.75	4.77	4.78	4.79
4	4.66	4.63	4.65	4.66	4.67	4.68	4.69	4.69	4.70	4.72
5	4.69	4.66	4.68	4.70	4.71	4.72	4.72	4.74	4.75	4.77
6	4.63	4.59	4.62	4.63	4.65	4.67	4.67	4.69	4.71	4.73

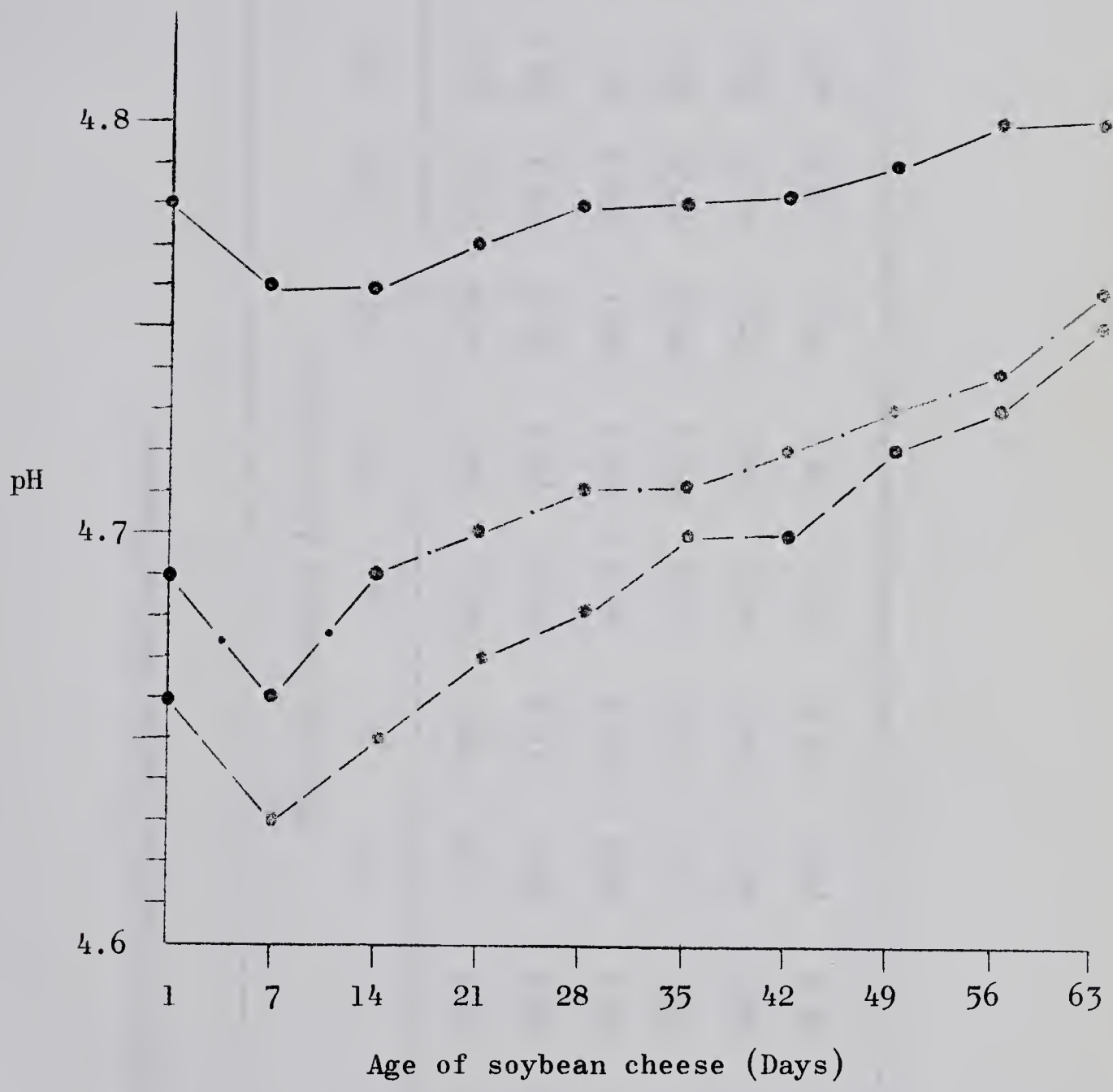


Figure 5. Changes in pH of soybean cheese during ripening

———————— Mean of data from cheese 1 and 2 (starter only)

——.——.—— Mean of data from cheese 3 and 4 (starter and rennet extract)

—— —— —— Mean of data from cheese 5 and 6 (starter and rennet extract plus skimmilk)

Table 10. Moisture content of soybean cheese during ripening.

Cheese No.	Moisture content (%) on day.									
	1	7	14	21	28	35	42	49	56	63
1	76.9	72.7	71.9	70.4	69.4	68.1	68.0	67.9	67.7	67.6
2	76.5	72.9	72.1	71.9	70.6	70.2	69.9	69.1	68.9	68.1
3	75.9	70.2	69.9	69.2	68.9	68.5	68.1	67.3	67.1	66.9
4	76.3	72.4	71.6	70.8	70.0	69.3	68.8	67.6	66.7	66.3
5	74.6	70.9	69.5	69.0	68.9	68.0	67.2	66.6	65.9	64.8
6	75.4	72.9	72.1	71.6	70.0	69.7	69.4	68.9	68.2	67.9

and gradual loss of moisture in all cheese examined. The heavy loss of moisture in the cheese during the first week of ripening is probably a result of the salting procedure.

Distribution of nitrogen during ripening

Protein decomposition in soybean cheese during ripening is shown in Table 11 and Figure 6. Each curve represents the mean of data from two cheese made under similar conditions. It can be seen that the water-soluble nitrogen content of the cheese prepared using soybean milk and starter only does not show any variation throughout ripening. However, the cheese containing rennet extract show a steady rise in water-soluble nitrogen content throughout the ripening period. The addition of skimmilk had very little or no effect on the degree of proteolysis in the cheese.

Total nitrogen content of all the cheese examined remained relatively constant throughout ripening. Cheese made with starter only and starter plus rennet extract contained 8.3% total nitrogen, whereas those made with the addition of skimmilk contained 8.5%.

Organoleptic judgement

Table 12 shows the characteristics of soybean cheese ripened 63 days at 20°C. The cheese made with soybean milk and starter only, remain fresh and elastic while those made with addition of rennet extract become gradually softer and mild. The values obtained by the use of the shear press indicate that the addition of rennet extract to the cheese markedly reduced the hardness. The softening is the result of the action of the proteolytic enzymes on the soybean protein. This is in agreement with the data on water-soluble nitrogen (Table 11).

Table 11. Protein decomposition in soybean cheese during ripening

Cheese No.	Water-soluble nitrogen (%) on day:									
	1	7	14	21	28	35	42	49	56	63
1	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31
2	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
3	0.45	0.84	1.18	1.46	1.67	1.84	1.95	2.10	2.23	2.34
4	0.49	0.91	1.25	1.57	1.70	1.92	2.05	2.14	2.34	2.44
5	0.35	0.77	1.12	1.46	1.74	1.88	1.98	2.14	2.30	2.40
6	0.38	0.84	1.22	1.53	1.81	1.95	2.10	2.23	2.37	2.47

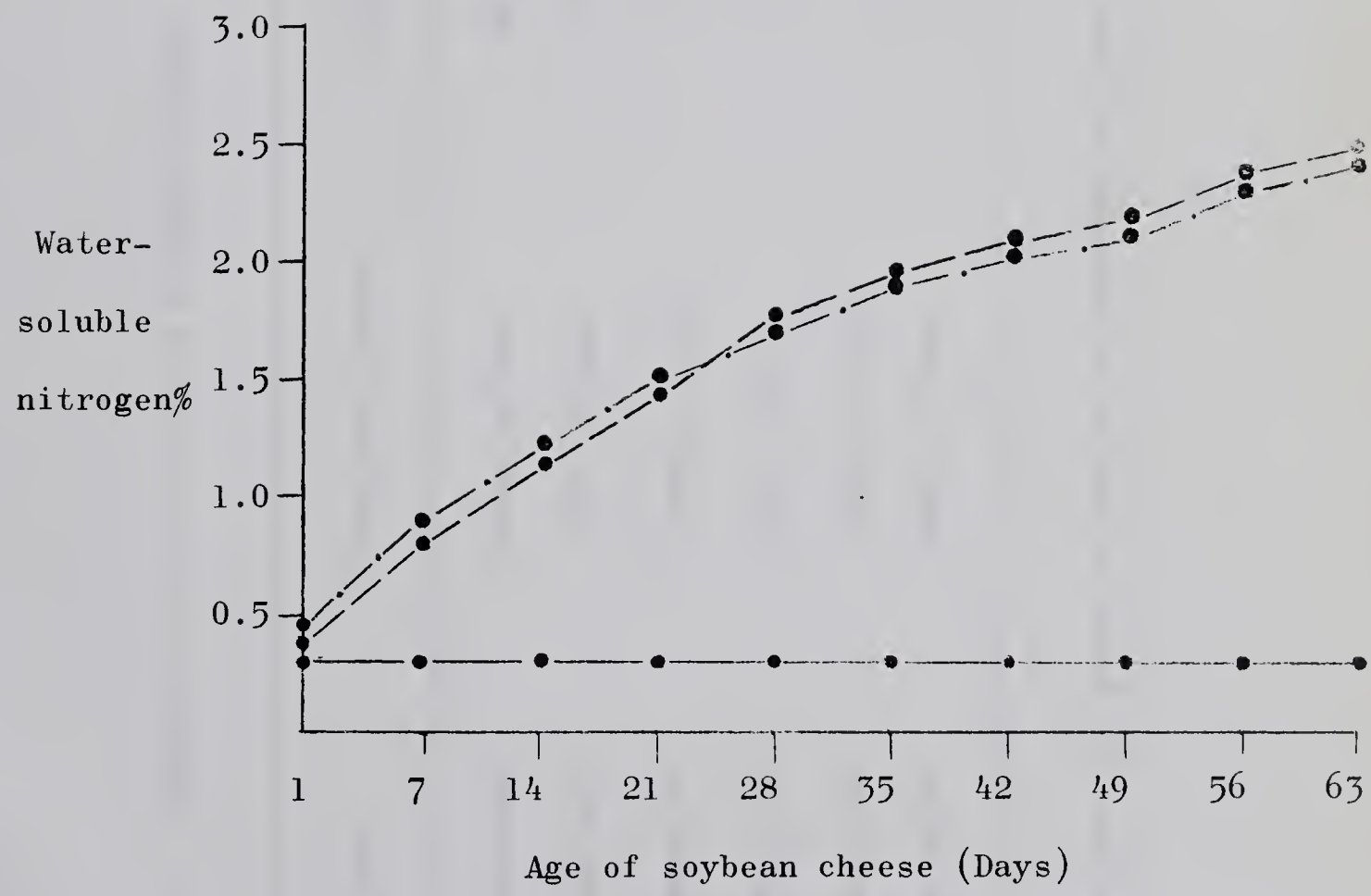


Figure 6. Protein decomposition in soybean cheese during ripening

———————— Mean of data from cheese 1 and 2 (starter only)

—— ·—— ·—— Mean of data from cheese 3 and 4 (starter and rennet extract)

—— —— Mean of data from cheese 5 and 6 (starter and rennet extract plus skimmilk)

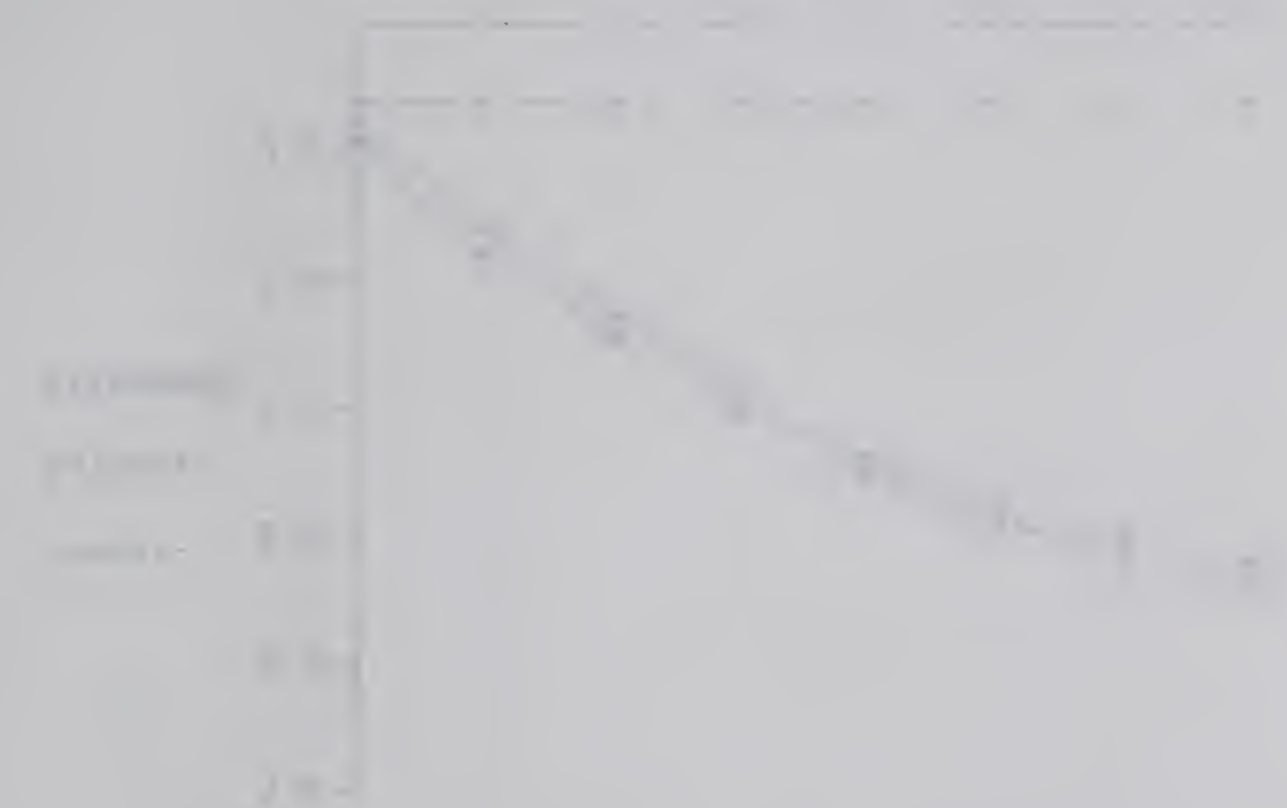


Table 12. Some organoleptic characteristics of ripened soybean cheese.

Cheese No.	Flavor	Body and Texture	Hardness*	
			1 day	63 days
1	Clean-fresh	Curdy, close	40	58
2	Clean-fresh	Curdy, close	42	57
3	Clean-mild	Crumbly, close	16	10
4	Clean-mild	Pasty, close	13	8
5	Clean-mild	Crumbly, close	21	14
6	Clean-mild	Pasty, close	18	9

* Value expressed as pounds per 100 grams of sample as measured by shear press.

DISCUSSION AND CONCLUSIONS

Soybean curd (tofu) and soybean cheese prepared by the conventional methods possess certain undesirable characteristics. The former is bland in flavor, difficult to handle and readily undergoes spoilage. The latter has a longer shelf life, but this is achieved only by the addition of large amounts of salt and alcohol. The prime reason for undertaking this work was to investigate other methods for the preparation of a product superior to tofu and conventional soybean cheese in terms of palatability, body and texture and shelf life.

Preliminary studies on the preparation of soybean curd using acetic acid, calcium sulfate and starter organisms indicated that there were variations in the recovery of protein. Approximately 12 - 13% more protein was recovered by the use of acetic acid than by either of the other two methods. The greater yield of protein by acid precipitation would appear to be outweighed by the objectionable characteristics of the curd. According to Hand et al. (1964), acid precipitated curd is very gritty, and as a result has never been acceptable for human consumption. Although the yield of protein by the other two methods was less, the resulting curd was superior in body and texture. The use of calcium sulfate, which is the traditional method of precipitation, results in a high concentration of calcium in the curd. Curd precipitated in this way has been recommended by Chiu and van Duyne (1961) as a good source of calcium in the diet in countries where cow's milk is unavailable or very expensive. Other

salts of calcium, for example calcium chloride, could be used for this purpose, however Watanabe et al. (1960) have found that the use of calcium sulfate results in the formation of a more gelatinous and smoother curd.

The moisture content and hardness of the curd prepared by the three methods showed marked variations. The lactic acid curd was the hardest and contained the least moisture. The softest curd and also the one containing the most moisture was produced by the use of calcium sulfate. Acetic acid gave a curd intermediate in these characteristics. As high moisture content of foods is generally conducive to the growth of micro-organisms, the lactic acid curd might be expected to be less subject to spoilage than the calcium sulfate curd.

These results show that curd produced from soybean milk by lactic acid fermentation contains less moisture and has a better body and texture than curd produced by either acid or salt precipitation.

Having demonstrated that a satisfactory curd could be prepared by means of a lactic acid fermentation of soybean milk, experiments were undertaken to reduce the time from the addition of starter to the cutting of the curd. Reduction of the time required to produce the curd would minimize the chance of contamination by undersirable micro-organisms and would lower the cost of production.

Rennin, pepsin and papain were added to the soybean milk to speed up coagulation, however none of the enzymes effected any changes in the time for coagulation to occur.

Skimmilk, when added to the soybean milk together with rennet extract and starter bacteria, reduced the coagulation time from just over three hours to two and a half hours. This is probably a result of the action of the rennet extract on the skimmilk. It is also possible that the skimmilk had a stimulating action on the growth of the starter bacteria, resulting in a more rapid utilization of fermentable substrates and increased acid production.

The determination of numbers of starter bacteria showed that multiplication occurred during the coagulation period and after pressing the curd and gradually decreased until at the end of seven weeks no starter bacteria could be detected. The decrease in numbers during ripening is probably a result of lack of fermentable substrates and the low ripening temperature.

The medium used for enumeration of the micro-organisms in the product was designed for the detection of proteolytic, acid-forming and alkali-producing species. Only acid-producing bacteria were found in all of the cheese examined. Lactobacilli, which are of prime importance during the ripening process of most cheese made from cow's milk, were not found in any of the cheese.

The water-soluble nitrogen content of cheese prepared using soybean milk and starter remained constant throughout the period of ripening, indicating that the starter bacteria did not possess proteolytic activity. From this it would appear that the main function of the starter bacteria is acid production.

As ripening progressed, the body of rennet-incorporated cheese gradually softened and became less elastic; its flavor became mild. These changes are probably the result of the solubilizing action of the proteolytic enzymes present in the rennet extract on soybean protein. It is likely that the products of proteolysis contribute to the flavor of the cheese. The inclusion of skimmilk in the rennet-incorporated cheese seemed to improve the flavor of the finished product. It is probable that the improved flavor is partially a result of the products formed by the enzymatic decomposition of casein as well as soybean protein.

Soybean cheese prepared from soybean milk using starter bacteria, rennet extract and skimmilk, would appear to be a good method for the conversion of raw soybeans to a palatable human food. The method of preparation gives rise to two by-products - the bean residue and whey. The bean residue left after aqueous extraction of soybean protein can be used as animal feed. According to Hackler et al. (1963), the residue has a high nutritive value approximating that of skimmilk. This suggests that as much as possible of this residue be left in soybean milk preparations designed for consumption by children in developing countries. Soybean whey is the product left after the separation of soybean protein from soybean milk. Because of its low solids content, recovery of whey is not economically feasible, and it is discarded. Because this practice increase the biological demand of sewage systems, use of soybean whey as a substrate for the production of high protein food products by fungal fermentation in submerged culture has been investigated by Falanghe et al. (1964).

About 4 to 6 g of mycelial protein per liter can be obtained from fermentation of soybean whey. Utilization of soybean whey by fungal fermentation may have an economic value in whey disposal and in the preparation of products of high protein content.

Further experiments are needed to reduce the time of coagulation and to improve the process of ripening, resulting in a more palatable product. In order to reduce the time of coagulation, a greater knowledge of soybean protein is required. Since several milk-clotting enzymes of both animal and vegetable sources fail to coagulate the soybean milk, investigation of microbial enzymes may be fruitful. As the addition of skimmilk resulted in a reduction of the time of coagulation from just over three hours to two and a half hours, a shorter coagulation time might be obtained if more skimmilk were added to the soybean milk. In the consideration that skimmilk is a very cheap material and is available in vast quantities, the use of large amounts of skimmilk in combination with soybean milk for the preparation of a new product with a better nutritive value might be expected.

The improvement of the palatability of the cheese might be brought about by the use of proteolytic micro-organisms together with Str. thermophilus. A Roquefort-type or Camembert-type cheese may also be obtained by the use of molds with Str. thermophilus. The molds which might play an important role in this connection are Penicillium roqueforti and Penicillium camemberti or species of the genus Mucor isolated from Chinese soybean cheese.

Soybean curd has to be freshly prepared and consumed daily because of the ease with which it undergoes putrefaction. The Chinese method of preparing soybean cheese results in a very salty product. The soybean cheese described in this work may be of considerable importance to those countries where soybean food products are readily acceptable and where high protein food products such as milk, meat, fish and eggs are very expensive.

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